Analytical Biochemistry **297,** 60–70 (2001)
doi:10.1006/abio.2001.5329, available online at http://www.idealibrary.com on IDEAL®

# A Simplified Reconstitution of mRNA-Directed Peptide Synthesis: Activity of the Epsilon Enhancer and an Unnatural Amino Acid[1]

Anthony C. Forster,*[,2] Herbert Weissbach,† and Stephen C. Blacklow*[,2]

**Department of Pathology, Brigham and Women's Hospital and Harvard Medical School, 75 Francis Street, Boston, Massachusetts 02115; and †Center for Molecular Biology and Biotechnology, Florida Atlantic University, 777 Glades Road, Boca Raton, Florida 33431*

Received March 13, 2001; published online August 30, 2001

**The study of the early events in translation would be greatly facilitated by reconstitution with easily purified components. Here, *Escherichia coli* oligopeptide synthesis has been reconstituted using five purified recombinant His-tagged *E. coli* initiation and elongation factors. Highly purified ribosomes are required to yield products with strong dependencies on the translation factors. Based on HPLC separation of radiolabeled translation products from an mRNA encoding a tetrapeptide, approximately 80% of peptide products are full length, and the remaining 20% are the dipeptide and tripeptide products resulting from pausing or premature termination. Oligopeptide synthesis is enhanced when a commonly used epsilon (*e*nhancer of *p*rotein *s*ynthesis *i*nitiation) sequence is included in the mRNA. The system incorporates a selectable, large, unnatural amino acid and may ultimately form the basis of a pure translation display technology for the directed evolution of peptidomimetic ligands and drug candidates. The recombinant clones can be exploited to prepare initiation factors and initiation complexes for structural studies, to study initiation and elongation in ribosomal peptide synthesis, and to screen for eubacterial-specific drugs.** 

***Key Words:*** **translation; reconstitution; *E. coli*; ribosome; initiation factor; elongation factor; tRNA; enhancer; epsilon; peptide; unnatural amino acid; biotin.**

The initiation of translation is a major control point in gene expression and is the target of a number of cytotoxic agents (1). Initiation is less well understood than elongation and termination in eubacteria (2–4), and initiation is much more complex in eukaryotes (5). It therefore seems likely that the study of the simpler eubacterial initiation process may be helpful both in the elucidation of general mechanistic principles employed in initiation and in the design of eubacterial-specific drugs.

mRNA-directed protein synthesis has been reconstituted using purified components from *Escherichia coli* (6–9), but in eukaryotes, translation is considerably more complex and essential translation factors are still being discovered (10). Both the formation and the competence of an fMet-tRNA$_i^{fMet}$:mRNA:70S ribosome initiation complex have also been tested stringently using subsets of the purified *E. coli* system in dipeptide (11) or tripeptide synthesis (12). The *in vitro* tripeptide system is highly dependent on two initiation factors (IF2 and IF3)[3] and on two of the three well-established

[1] This work was supported by NIH Grants T32-HL07627 (partly to A.C.F.), K08-CA80833 (to A.C.F.), and R01-HL61001 (to S.C.B.); Massachusetts Department of Public Health Grant 34089PP1030 and DOD Idea Award DAMD17-00-1-0163 (to S.C.B.); and by Hoffmann-La Roche (to H.W.). S.C.B. is a Pew Scholar in the Biomedical Sciences.

[2] To whom correspondence may be addressed. A.C.F. Fax: (617) 732-7449. E-mail: aforster@rics.bwh.harvard.edu. S.C.B. Fax: (617) 264-5296. E-mail: sblacklow@rics.bwh.harvard.edu.

[3] Abbreviations used: bK, biotin-labeled-lysine; DEAE, diethylaminoethyl; DTT, dithiothreitol; EF-G, elongation factor G; EF-Ts, elongation factor Ts; EF-Tu, elongation factor Tu; epsi, *e*nhancer of *p*rotein *s*ynthesis *i*nitiation (epsilon) sequence; fM, formylmethionine; H, N-terminal His$_6$-tag; IF1, initiation factor 1; IF2, initiation factor 2; IF2-2, internal translation initiation protein product of the IF2 gene; IF3, initiation factor 3; IPTG, isopropyl $\beta$-D-thiogalactoside; K, lysine; M, methionine; MetRS, methionyl-tRNA synthetase; NTA, nitrilotriacetic acid; PAGE, polyacrylamide gel electrophoresis; PEG, polyethylene glycol; $P_i$, inorganic phosphate; S-D, Shine and



0003-2697/01 $35.00

elongation factors (EF-Tu and EF-G, but not EF-Ts) and is stimulated by addition of the third initiation factor (IF1). A more stringent requirement for IF1 has, nevertheless, been demonstrated *in vivo* where all three initiation factors are necessary for cell viability (13–15). Interestingly, internal initiation of translation from the IF2 gene produces a form that is 18 kDa shorter, now termed IF2-2 (16), present at a concentration half that of IF2 *in vivo* (17). Although the loss of either form is not lethal, both forms are necessary for maximal growth and may have roles in processes other than translation initiation (17).

The lack of availability of clones designed for high-level overexpression of tagged initiation factors has made the reproducible preparation of large quantities of highly purified factors to reconstruct a purified initiation system a major technical challenge. Indeed, many studies have relied on gifts of key components. In order to overcome this problem, we subcloned and overproduced all three *E. coli* initiation factors with $His_6$ tags and tested the activity of each factor in a purified translation system. This system has been used to investigate the role of an mRNA sequence element derived from phage T7 known as epsilon (*e*nhancer of *p*rotein *s*ynthesis *i*nitiation (18)), the activity of which is controversial (19), and to test for unnatural amino acid incorporation.

## MATERIALS AND METHODS

*Construction of plasmids for the overexpression of His-tagged E. coli IF1, IF2, and IF3 proteins.* *E. coli* initiation factor coding sequences, each containing an insertion of six histidines immediately after the N-terminal methionine, were synthesized by PCR from published plasmids and subcloned into a vector derived from pET24a (Novagen). Plasmid pXR201 containing the native IF1 sequence encoded by an artificial sequence of *E. coli*-preferred codons (instead of *infA* codons) was kindly supplied by R. Spurio and C. Gualerzi (20) and subcloned to give pAF1H. Plasmid pSL4 containing the native IF2 sequence encoded by *infB* was kindly supplied by S. Laalami and M. Grunberg-Manago (21) and subcloned to give pAF2H. Plasmid pDD1 containing the native IF3 sequence encoded by *infC* was kindly supplied by N. Brot and I. Schwartz (22) and subcloned to give pAF3H. The sequences of the three subclones, characterized by a combination of restriction digests and sequence analyses, begin (ligation sites underlined) with TATACA/TATG$(CAC)_6$ before the second amino acid; the final amino acid is followed by the sequence TAAG/AATTCGAGCTCCGTCGA/42 bp deletion/AGATCC, and the remainder of the sequence is from pET24a.

*Overexpression and purification of His-tagged E. coli translation factor proteins.* Plasmid pHTA7 (in *E. coli* BL21(DE3)) encoding His-tagged *E. coli* EF-Tu, containing a $His_6$ sequence inserted between the first two codons of *TufA*, was kindly supplied by Y.-W. Hwang and D. Miller (unpublished; see 23). Plasmid pRSET/EF-G(His) (in *E. coli* BL21(DE3) cells together with the pLysS plasmid) encoding His-tagged *E. coli* EF-G (EF-GH), containing an N-terminal extension of about 30 amino acids including $His_6$, was kindly supplied by A. Savelsbergh and W. Wintermeyer (24). Expression of our three initiation factor subclones (in *E. coli* BL21(DE3)pLysS; Novagen) and the two supplied clones was induced with IPTG. All the factors were expressed predominantly in the soluble cellular fractions and purified by step elution from Ni-NTA agarose columns using standard protocols (Qiagen), except that 10 $\mu$M GDP was included up to the last dialysis step for EF-TuH. All factors were dialyzed against buffer A (10 mM Tris–HCl, pH 7.4, 1 mM $MgCl_2$, 1 mM DTT). Precipitated IF3H was recovered by redissolving in 5 M urea (25), diluted, and then dialyzed against buffer A containing 100 mM $NH_4Cl$. In contrast to the extensive proteolytic degradation observed by others during the overexpression and purification of native IF2 (26), recombinant IF2H was not proteolytically labile. All factors were stored at −80°C. They were thawed many times without loss of activity, except for EF-TuH, which was stored at 4°C after thawing and used within a few weeks.

*Purification of E. coli ribosomes (27).* SOLR cells (Stratagene) grown to midlog phase were resuspended in buffer B (60 mM KOAc, 14 mM $Mg(OAc)_2$, 10 mM Tris–HOAc, 1 mM DTT, pH 7.9), sonicated, and centrifuged at 10,000*g*. The supernatant was centrifuged at 30,000*g*, and the resulting supernatant was then centrifuged at 150,000*g*. The ribosome pellet was washed by stirring in buffer B containing 1 M $NH_4Cl$ at 4°C overnight and then repelleting at 150,000*g*. The washing was repeated twice more to give 3× washed ribosomes which were resuspended in buffer C (10 mM $Mg(OAc)_2$, 1 mM Tris–HCl, pH 7.4, 1 mM DTT) and stored at −80°C. Ribosomes washed only once gave poor factor dependencies in translation assays of the type illustrated in Fig. 5 (data not shown).

*Synthesis of mRNAs.* mRNAs were transcribed (28) from synthetic oligodeoxyribonucleotides (Research Genetics) and purified by gel electrophoresis (29). Because the templates are relatively long, extended deprotection times were necessary (12 h) following the synthesis of the blocked oligonucleotides to enable optimal transcription.

*Preparation of aminoacyl tRNAs.* Pure *E. coli* tRNA isoacceptors were from Subriden RNA. Each

Dalgarno ribosome binding site; T, threonine; TFA, trifluoroacetic acid; THF, tetrahydrofolate; ThrRS, threonyl-tRNA synthetase; V, Val; ValRS, valyl-tRNA synthetase.

isoacceptor was prepared by the manufacturer from *E. coli* total tRNA (Plenum) using three column chromatography steps. The first fractionation used BD cellulose; the second, DEAE–Sephadex at pH 7; and the third, DEAE–Sephadex at pH 5 or Sepharose. Natural aminoacyl tRNAs were prepared from these isoacceptors as follows. High-specific-activity [$^3$H]fMet-tRNA$_i^{fMet}$ (24,000 dpm/pmol in Table 1) and low-specific-activity [$^3$H]- or [$^{35}$S]fMet-tRNA$_i^{fMet}$ (a few hundred dpm/pmol; used for all other studies) were prepared as described (11) using MetRS (kindly supplied by G. Chen), Met-tRNA$_i^{fMet}$ formyltransferase (kindly supplied by N. Brot), and $N^{5,10}$-methenyl THF (synthesized using $N^{10}$-formyl THF synthetase kindly supplied by K. Kounga and R. Himes). [$^{14}$C]Thr-tRNA$_3^{Thr}$ (510 dpm/pmol) and [$^3$H]Val-tRNA$_1^{Val}$ (21,000 dpm/pmol in Figs. 5–7; 28,000 dpm/pmol in Figs. 4 and 8) were aminoacylated as described (11) with ThrRS (kindly supplied by I. Schwartz), ValRS (kindly supplied by J. Horowitz), or, for the lower specific activity Val-tRNA, a tRNA-free preparation of total *E. coli* synthetases partially purified from an 150,000*g* supernatant by step elution with 0.3 M KCl from DEAE–Sepharose (see 30).

Biotin-labeled Lys-tRNA$^{Lys}$ (Transcend tRNA) was purchased from Promega. This material was prepared by the manufacturer from *E. coli* total tRNA by charging with lysine using a crude preparation of total synthetases, enriching for Lys-tRNA$^{Lys}$ by ion-exchange chromatography, and chemical coupling to biotin via an uncharged, 13-carbon-long spacer.

*Assay of His-tagged E. coli translation factor proteins.* Initiation factor assays (27) used custom-synthesized ApUpG RNA template (TriLink Biotechnologies). A mixture containing 0.95 $\mu$M IF1H, 0.15 $\mu$M IF2H, 0.78 $\mu$M IF3H, 3× washed ribosomes at 0.029 $A_{260}/\mu$l (33 nM estimated to be active in translation; see below), 0.29 $\mu$M [$^3$H]fMet-tRNA$_i^{fMet}$, 150 $\mu$M AUG, 0.4 mM GTP in 50 mM Tris–HCl, pH 7.4, 100 mM $NH_4Cl$, 5 mM $Mg(OAc)_2$, and 2 mM DTT was incubated at 37°C for 10 min. After dilution, the mixture was rapidly filtered through nitrocellulose to separate initiation complex-bound fMet-tRNA$_i^{fMet}$ from unbound species. Reactions that lacked template were used as controls for nonspecific binding (29% of maximal dpm for Table 1; 9% of maximal dpm using higher (saturating) concentrations of ribosomes that bound 70% of [$^3$H]fMet-tRNA$_i^{fMet}$). Because of the high affinity of EF-Tu for EF-Ts, EF-Ts activity assays were performed (31) with EF-TuH, indicating a copurification level of about 2%. EF-TuH activity was measured by GDP binding (31), and EF-GH activity was measured by a ribosome-dependent GTPase assay (31).

*Translations.* The components of translation mixes were adapted from published work (11, 12). Five times premix buffer was prepared from a solution containing 180 mM Tris–HOAc (pH 7.5), 50 mM sodium 3,3-dimethyl-glutarate (pH 6.0), 180 mM $NH_4OAc$, 10 mM DTT, 140 mM potassium phosphoenolpyruvate (pH 6.6), 195 mM KOAc, and 4 mM spermidine · 3HCl by adjusting it to pH 6.8 with NaOH. As a representative example, the mixture in the translation of the MTTV mRNA template (Fig. 6; 30 $\mu$l total volume) included 1× premix buffer, 9.5 mM $Mg(OAc)_2$, 1 mM GTP, 14 ng/$\mu$l pyruvate kinase, 4.3% PEG 8000, 0.95 $\mu$M IF1H, 0.15 $\mu$M IF2H, 0.78 $\mu$M IF3H, 3.1 $\mu$M EF-TuH, 0.88 $\mu$M EF-GH, 3× washed ribosomes at 0.029 $A_{260}/\mu$l (33 nM estimated to be active in translation; see below), 0.29 $\mu$M [$^3$H]fMet-tRNA$_i^{fMet}$, 0.58 $\mu$M [$^{14}$C]Thr-tRNA$_3^{Thr}$, 0.29 $\mu$M [$^3$H]Val-tRNA$_1^{Val}$, and 1 $\mu$M mRNA. This mixture was incubated at 37°C for 50 min, although some translations were as short as 1 min (Fig. 4). The setup for some translations also included preincubations at 37°C for 10 min of an initiation mix (GTP, IF1H, IF2H, IF3H, ribosomes, [$^3$H]fMet-tRNA$_i^{fMet}$, and mRNA) and an elongation factor mix (GTP, EF-TuH, EF-GH, [$^{14}$C]Thr-tRNA$_3^{Thr}$, and [$^3$H]Val-tRNA$_1^{Val}$ (Fig. 4)).

For HPLC analysis (Fig. 6), peptides and amino acids were released from tRNAs by addition of 1 M NaOH (6 $\mu$l) and incubation at 37°C for 20 min. Unlabeled marker peptides (Research Genetics) were then added (18 $\mu$l with a combined peptide concentration of 10 $\mu$g/$\mu$l in $H_2O$), the solution was acidified with glacial acetic acid (5 $\mu$l) and microcentrifuged, and the supernatant was then microcentrifuged through a Microcon 10 ultrafiltration device (10-kDa cutoff; Amicon). A portion of the filtrate (20 $\mu$l) was loaded onto a C18 reversed-phase column (Vydac) and eluted with a 0 to 31.5% $H_2O$/MeCN gradient containing 0.1% TFA at 1 ml/min with detection by absorbance at 229 nM and scintillation counting of 42-drop fractions using a dual-labeled dpm program (Packard).

For analysis of the fMTV and fMVT syntheses, formylated peptides and formyl methionine were separated from unformylated amino acids by base hydrolysis, acidification, and cation-exchange chromatography on minicolumns (32).

*Control translations.* Additional translations were performed to assess the dependency on components other than the translation factors. Omission of [$^3$H]fMet-tRNA$_i^{fMet}$ or [$^{14}$C]Thr-tRNA$_3^{Thr}$ abolishes fMTV synthesis from mRNA MTV, based on incorporation of [$^3$H]Val into product. In the experiment omitting [$^{14}$C]Thr-tRNA$_3^{Thr}$, addition of uncharged tRNA$_3^{Thr}$ does not reconstitute measurable fMTV synthesis, demonstrating a lack of ThrRS activity under translation conditions, as expected for a purified system (as a further control, addition of uncharged tRNA$_3^{Thr}$ was not inhibitory to fMTV synthesis). Omission of [$^3$H]Val-tRNA$_1^{Val}$ abolishes fMVT synthesis from mRNA MVT (Fig. 3), based on incorporation of [$^{14}$C]Thr-tRNA$_3^{Thr}$ into product. The threonine/valine (T/V) ratios in the mRNA MVT and mRNA mMV (Fig. 3) products are

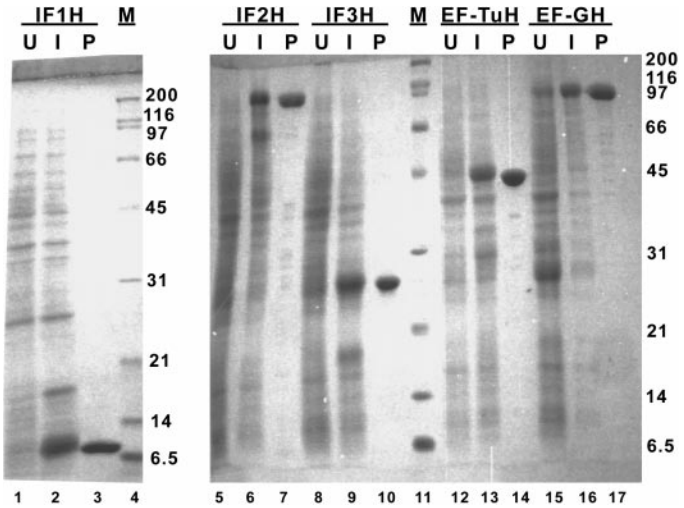


**FIG. 1.** Overexpression and purification of five His-tagged *E. coli* translation factors from *E. coli*. After SDS–PAGE on 15% gels, samples were stained with Coomassie blue. U, uninduced total cells; I, IPTG-induced total cells; P, purified protein eluted from $Ni^{2+}$ beads; M, molecular weight marker proteins (sizes indicated in kDa).

about 1.0 and 0, respectively, as expected. Omitting ribosomes gives the lowest background radioactivity measurements, and translations lacking mRNA do not accumulate formylated peptide products with time. Omitting rabbit muscle pyruvate kinase ((33); the protein from Sigma migrates as a single major band on SDS–PAGE) decreases the yield of product by 50%. Omitting PEG decreases the yield by 25%, and the standard concentration of $Mg^{2+}$ (9.5 mM) is optimal. Although RNase activity is high in crude *E. coli* translation systems (34), it is undetectable in the purified system as judged by urea–PAGE of radiolabeled mRNA before and after translation.

## RESULTS

*Subcloning, overexpression, and purification of His-tagged E. coli translation factors.* Published clones for the expression of *E. coli* IF1, IF2, and IF3, though useful in numerous initiation studies, are for untagged factors that cannot be affinity purified and, with one exception, are thermally inducible (20–22, 26, 35). An important consideration for IF3 overexpression is the presence of the rare AUU initiation codon (35). Initiation codon pairing with fMet-$tRNA_i^{fMet}$ is directly proofread by IF3 (36), thereby enabling IF3-mediated feedback repression of translation of its own gene *in vivo* (35). Thus, to increase the expression levels and to simplify purification of all three initiation factors, we replaced the initiation codons with an $AUG(CAC)_6$ sequence by PCR and subcloned the coding sequences into a pET-derived expression plasmid (see Materials and Methods). The resulting His-tagged clones were nontoxic and overproduced the factors (termed IF1H, IF2H, and IF3H) at very high levels in the soluble fraction of the lysate (Fig. 1, lanes 1, 2, 5, 6, 8, and 9). In addition, *E. coli* EF-Tu and EF-G with N-terminal $His_6$ tags (termed EF-TuH and EF-GH) were overexpressed using available clones (Fig. 1, lanes 12, 13, 15, and 16; unpublished; (23, 24)). Gel analysis of the $Ni^{2+}$-purified initiation and elongation factors is also shown in Fig. 1 (lanes 3, 7, 10, 14, and 17). IF2H, IF3H, EF-TuH, and EF-GH comigrated with samples of the authentic *E. coli* proteins (data not shown), and IF1H had the expected electrophoretic mobility based on comparison with molecular weight markers (Fig. 1, lanes 1–4). IF2-2 was probably overexpressed together with IF2H (Fig. 1, lane 6), but, because its synthesis by internal translation initiation (17) would not incorporate a $His_6$ tag, it did not copurify with IF2H (Fig. 1, lane 7). The minor induction band at approximately 18 kDa in lanes 2 and 9 of Fig. 1 corresponds to T7 lysozyme, a product of the pLysS plasmid, as deduced by N-terminal sequencing (data not shown).

*Dependencies of His-tagged initiation factors in an initiation assay.* The activity and purity of the three initiation factors were measured by ribosome:fMet-$tRNA_i^{fMet}$:ApUpG trinucleotide complex formation (Fig. 2, top row). Dependencies of the His-tagged initiation factors in initiation complex formation with the 3× salt-washed ribosomes were comparable to those reported for native factors (Table 1; 27, 37). The variation in IF3 dependency may result from different amounts of free 30S ribosomal subunits in the different 70S ribosome preparations because IF3 does not strongly

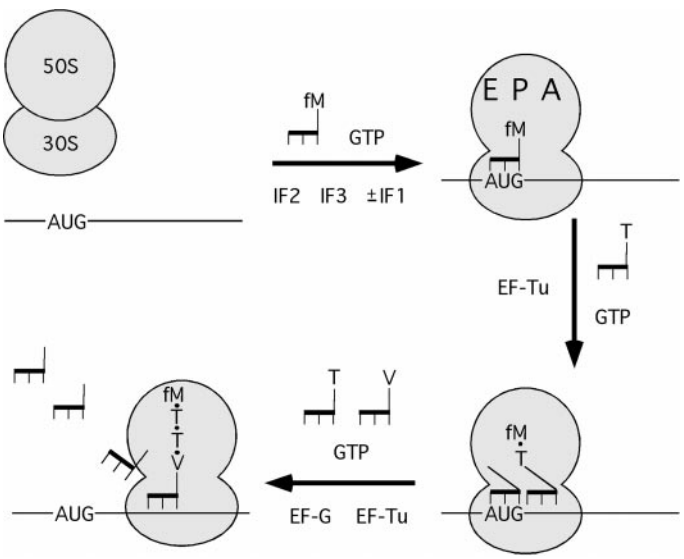


**FIG. 2.** Schematic illustrating steps in ribosome-directed oligopeptide synthesis. The three enzymatic reactions depicted by arrows are initiation (top), the first elongation step (right), and subsequent translocation and elongation steps (bottom). Peptide products can be released from the peptidyl-tRNAs by base-catalyzed hydrolysis for analysis. Products GDP and $P_i$ are not shown. E, exit site; P, peptidyl site; A, aminoacyl site.

**TABLE 1**

Factor Dependencies for Initiation Complex Formation

| Initiation factor omitted | Initiation complex[a] | | |
|---|---|---|---|
| | His-tagged[b] | Native (from Ref. 27)[c] | Native (from Ref. 37)[d] |
| None | 100 | 100 | 100 |
| IF1 | 48 | 61 | 36 |
| IF2 | 0 | 4 | 7 |
| IF3 | 7 | 17 | 48 |

[a] % Maximal binding of $^3$H-labeled fMet-tRNA$_i^{fMet}$ to ribosomes in the presence of GTP.

[b] Performed with purified His-tagged initiation factors, ApUpG template, and 5 mM $Mg^{2+}$ (see Materials and Methods). The maximum concentration of fMet-tRNA$_i^{fMet}$ specifically bound into initiation complexes was 19 nM.

[c] Performed with purified native initiation factors, poly(U:G) (3:1) template, and 10 mM $Mg^{2+}$.

[d] Performed with purified native initiation factors, ApUpG template, and 5 mM $Mg^{2+}$.

stimulate initiation complex formation when free 30S subunits are substituted for 70S ribosomes in the assay (38). Given that initiation factors tend to copurify with ribosomes (27), the results also attest to the purity of the ribosomes. Substitution of the AUG trinucleotide template with the MTTV mRNA template (Fig. 3; described below) at a much lower concentration (0.3 $\mu$M) enables complex formation with equivalent yield (data not shown).

*Dependencies of His-tagged factors in tripeptide synthesis.* We next tested whether initiation complexes were competent to undergo elongation in a purified translation system using the components depicted in Fig. 2. The mRNA design we selected (39) resembles those used in recent purified *in vitro* translation systems (40–42) by containing an optimal Shine–Dalgarno sequence, but it differs by also containing an epsilon translational enhancer (18). Although the epsilon sequence is not necessary for di- and tripeptide synthesis from native and artificial *E. coli* mRNAs in purified systems (40–43), the potential influence of epsilon has not yet been tested in a purified system. Our design (Fig. 3) has the advantage of encoding mRNAs short enough to be synthesized directly from a single long synthetic DNA template hybridized to a standard 18-mer oligodeoxyribonucleotide without the need for cloning (28). Translations of the templates are completed when the ribosomes translocate to a codon for which there is no supplied cognate aminoacyl tRNA (43).

As shown in Fig. 4, reconstitution of oligopeptide synthesis with all of the components shown in Fig. 2 using 3× salt-washed ribosomes and the MTV mRNA (Fig. 3) results in the synthesis of fMTV, as judged by the incorporation of $^3$H-labeled valine into the isolated peptide products. When the time course of synthesis is begun by combining a preincubated initiation mix with a preincubated elongation factor mix (triangles in Fig. 4; see legend), there is a rapid initial burst of product

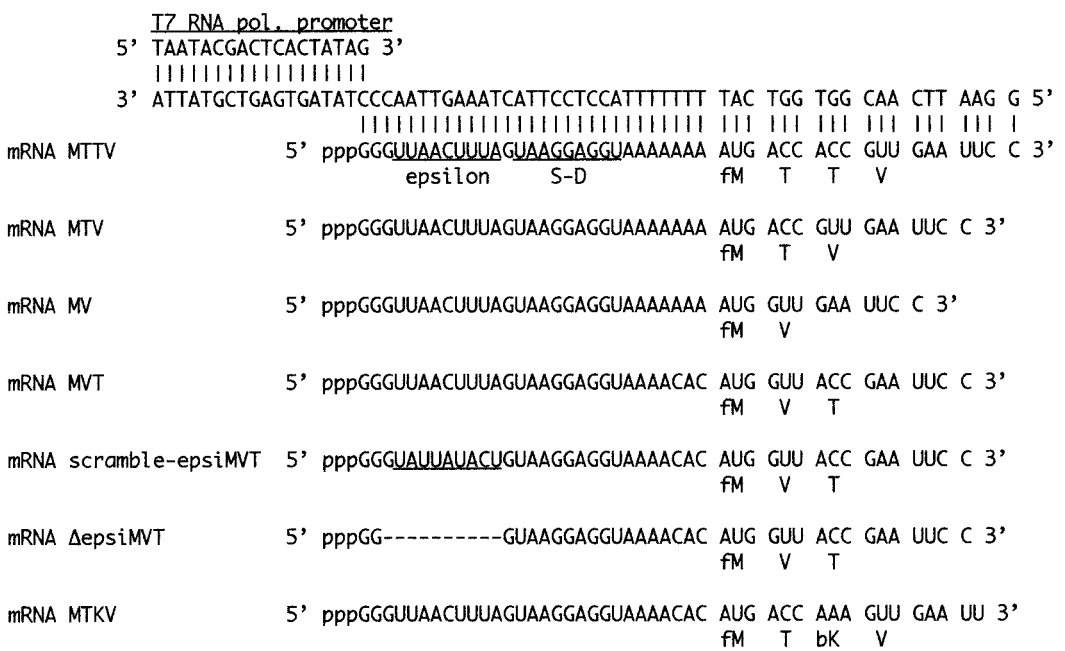


**FIG. 3.** mRNA templates used in this study. The DNA primer–template pair used to synthesize the longest mRNA is illustrated at the top. The predicted translation products from our purified system are also shown (aminoacyl tRNAs for the 3′ terminal codons GAA (Glu) and UUC (Phe) were not used). bK, biotin-labeled lysine. S-D, Shine and Dalgarno ribosome binding site.

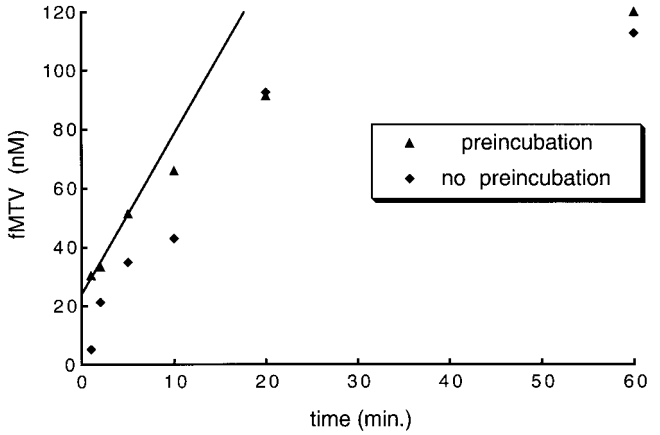


**FIG. 4.** Characterization of oligopeptide synthesis rates from mRNA MTV in a purified His-tagged translation system. fMTV was measured by [$^3$H]valine incorporated into peptide products in translations containing IF1H, IF2H, IF3H, EF-TuH, EF-GH, and 0.020 $A_{260}/\mu$l ribosomes. Triangles, translations were started by mixing preincubated initiation components with preincubated elongation components. Squares, translations were started by transferring the translation mix from 0 to 37°C. Aliquots were terminated with NaOH at the indicated times beginning at 1 min. Peptide product dpm was calculated by subtracting dpm obtained in aliquots terminated before 37°C incubation. Individual data points from representative experiments are plotted, with variations estimated to be less than 20%. A tangent line to the preincubation reaction curve is drawn to estimate the steady-state rate (44).

synthesis within the first minute and a slower rate of synthesis at steady state. This time course is consistent with initiation during preincubation, very rapid elongation upon mixing, and a rate-limiting recycling step when using limiting active ribosome concentrations in the absence of release factors (44). By extrapolation of the steady-state rate to $t = 0$, we estimate that 24 nM ribosomes are maximally active in translation (44), which corresponds to 5% of the ribosomes (assuming 23 pmol/$A_{260}$ unit). While a higher percentage of active ribosomes may be prepared by reducing the number of salt washes, such ribosomes are significantly contaminated with translation factors, as judged by factor dependencies (see Materials and Methods), so there appears to be a trade off between ribosome activity and purity. Without the 10-min preincubations (squares in Fig. 4; see legend), there is little product synthesis within the first minute, and synthesis is fairly linear with time (see also Fig. 7), as previously published (12). This rate of synthesis is consistent with the known slow rate of initiation complex formation in the absence of preincubation (44, 45).

We chose the assay without preincubation to compare the activities of our His-tagged factors and the purity of our ribosomes with published values for factor dependency in purified systems because the published conditions lack a preincubation step (12). Both original and more recent purified systems (42, 43) used high concentrations of translation components by comparison to aminoacyl tRNA substrates; similar conditions were used here. These conditions are also consistent with our ultimate goal of maximizing conversion of substrate to product without mRNA turnover for ribosome display (46). For directed *in vitro* evolution, it is desirable to minimize turnover of an mRNA because this leads to an uncoupling of genotype (mRNA) from phenotype (peptide; see Discussion). Figure 5 illustrates the dependency on each factor necessary for *in vitro* translation (IF1 was omitted in this experiment), based on the incorporation of $^{14}$C-labeled threonine and $^3$H-labeled valine into peptide products. The complete system yielded peptide products with a T/V ratio of 1.0, as expected for fMTV synthesis. Omission of any one factor dramatically reduces fMTV synthesis (Fig. 5), giving dependencies comparable to those reported for the native factors (11, 12). Residual synthesis in the absence of a factor may represent reported factor-free translation (47). The omission of translation factor EF-GH switched translation from tripeptide synthesis to fMT dipeptide synthesis (Fig. 5), consistent with the known primary role of EF-G in translocation. Although synthesis of dipeptides does not require addition of IF1 (11), and although the inclusion of IF1H in our translations does not lead to a dramatic increase in overall yield, IF1H does stimulate the rate of fMTV synthesis 2.5-fold during the first few minutes of translations performed without preincubation (data not shown), consistent with previously reported studies with native IF1 (11). Thus, the translation results confirm and extend the findings from the initiation assay (Table 1). Additional control translations omitting other macro-

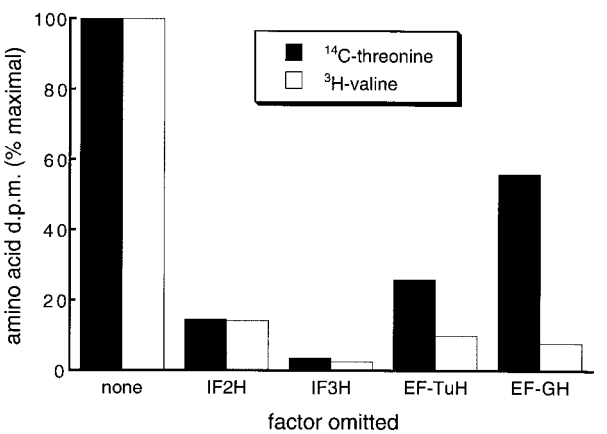


**FIG. 5.** Translation factor dependencies in the purified translation system with mRNA MTV. Light bars, [$^3$H]valine incorporated into peptide products (a measure of fMTV) shows strong dependencies on IF2H, IF3H, EF-TuH, and EF-GH (IF1H was omitted from these translations; see Materials and Methods). Dark bars, [$^{14}$C]threonine incorporated into the same products (a combined measure of fMT and fMTV). Peptide synthesis in a 30-min translation started by transfer from 0 to 37°C was calculated by subtracting dpm obtained in a control reaction lacking mRNA (1.3% of maximal dpm) from total dpm. The maximum concentration of synthetic product obtained was 0.12 $\mu$M for both T and V.

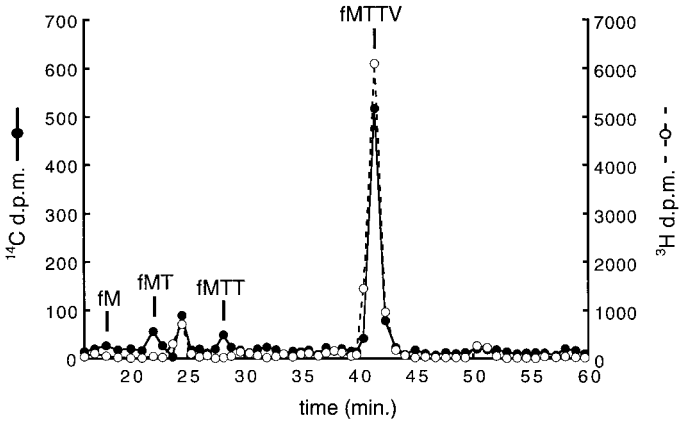


**FIG. 6.** HPLC analysis of products produced from the MTTV mRNA template. The peptide products of a dual-labeled translation were first released from the tRNAs with base and then mixed with unlabeled marker peptides. The mixture was acidified, microcentrifuged to remove insoluble material, and microcentrifuged through a 10-kDa filter before injection for analysis (see Materials and Methods). The elution positions of marker peptides are indicated above the chromatogram. Filled circles, [$^{14}$C]threonine total dpm. Open circles, [$^{3}$H]valine total dpm in the same fractions. The amount of product synthesized was 2.1 pmol in 30 $\mu$l (70 nM).

molecular components of the translation system give the expected dependencies (see Materials and Methods).

*Tetrapeptide synthesis.* We next investigated the suitability of this system for the synthesis of tetrapeptides to show that all steps of initiation and elongation could occur. In contrast to tripeptide synthesis, tetrapeptide synthesis is not possible without dissociation of deacylated tRNA from the exit site of the ribosome (48). Using a template encoding the tetrapeptide fMTTV (Fig. 3), synthesis of dual-labeled products was assessed by reversed-phase HPLC (Fig. 6). Radioactive peptide products were identified based on their comigration with chemically synthesized nonradioactive standards. The predominant radioactive peak in the peptidyl separation range corresponds to the fMTTV tetrapeptide (80–85% of the $^{14}$C or $^{3}$H radioactivity in this range) with a T/V ratio close to that expected. Two minor peaks correspond to the pausing or premature termination products fMT and fMTT, with no $^{3}$H incorporated, as expected. fMT and fMTT together contain 12% of the $^{14}$C radioactivity in the peptidyl range, equivalent to 20% of the combined products fMTTV, fMTT, and fMT on a molar basis. The remaining two minor peaks (at 24 and 50 min) presumably correspond to derivatives of fMTTV (e.g., methionine oxidation products or unformylated peptide), other peptidyl products, and/or nonpeptide radioactive contaminants. Thus, the purified His-tagged tetrapeptide translation system is predominantly, but not completely, processive with yields of full-length tetrapeptide products equal to 80% of the peptide products. This detailed analysis extends previous observations on the processivity of oligopeptide synthesis in purified systems (9, 12).

*Effects of upstream mRNA mutations on oligopeptide synthesis.* This series of experiments addresses the specific upstream mRNA sequence requirements needed for ribosome-mediated oligopeptide synthesis. Alteration of the nucleotides between the Shine–Dalgarno (S-D) sequence and the initiation codon can have an unpredictable effect on translation (49). In the MV, MTV, and MTTV mRNAs, this region of the template occurs within a string of eight adenines (Fig. 3) that have the potential to cause slipping of DNA and RNA polymerases (28) and to interfere with purification of 3′-poly(A)-tagged mRNAs on poly(dT) beads. Therefore, the effect of interrupting the poly(A) sequence with two A to C mutations was tested. These substitutions in the MVT mRNA (Fig. 3) have minimal effects on transcription and translation efficiencies (data not shown). The effect of mutations in the long S-D sequence is yet to be evaluated in our system.

An unresolved question in translation initiation is the influence of the epsilon sequence in a purified system. Therefore, the effect of scrambling or deleting epsilon was determined using the mRNAs scramble-epsiMVT and ΔepsiMVT (Fig. 3) and template concentrations (1 $\mu$M) that are saturating for mRNA MVT (data not shown) under conditions where initiation should be rate-limiting (see Fig. 4). Figure 7 shows that scrambling of the U-rich epsilon sequence results in decreased fMVT synthesis, and deletion of the sequence results in a 5-fold decrease in the initial rate of product synthesis. Concentrations of mRNA ΔepsiMVT

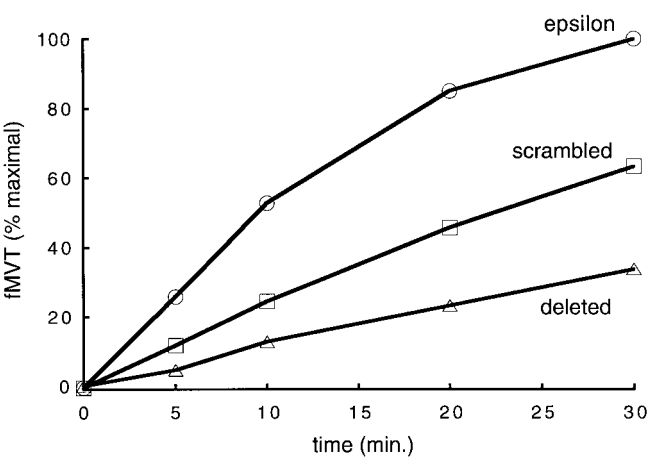


**FIG. 7.** Inclusion of the epsilon sequence enhances the synthesis of oligopeptide product in a purified translation system. Comparison between rates of fMVT synthesis from mRNAs MVT (circles), scrambled-epsiMVT (squares), and ΔepsiMVT (triangles). Dual-labeled translations containing IF1H, IF2H, IF3H, EF-TuH, and EF-GH were analyzed as described in the legend to Fig. 5. The maximum concentration of oligopeptide synthesized in 30 min was 0.25 $\mu$M using 0.5 $\mu$M of each aminoacyl tRNA.

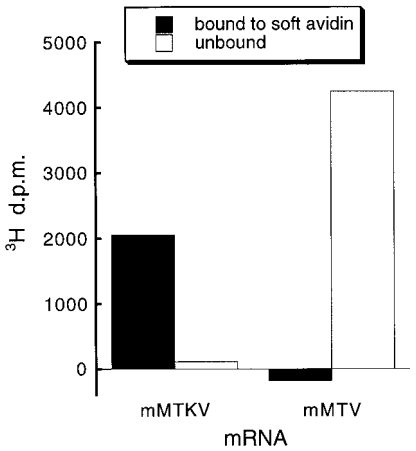


**FIG. 8.** Synthesis and selection of peptides containing an unnatural amino acid using the purified His-tagged translation system. Translation mixes containing biotin-labeled Lys-tRNA$^{Lys}$, fMet-tRNA$_i^{fMet}$, Thr-tRNA$_3^{Thr}$, and [$^3$H]Val-tRNA$_1^{Val}$ substrates and either mRNA MTKV or MTV (see Fig. 3 for translation products) were incubated at 37°C for 30 min. The peptides and amino acids were released from the tRNAs and ribosomes with base and neutralized, and the mixtures were incubated with soft avidin beads to bind biotin-containing molecules. The beads were washed four times to remove unbiotinylated molecules before counting bound $^3$H (dark bars, a measure of products containing biotin-labeled lysine covalently linked to [$^3$H]valine). The pooled washes were filtered, acidified, and passed through a cation-exchange column to count unbound $^3$H (light bars, a measure of formylated peptide products containing [$^3$H]valine without biotin-labeled lysine or lysine). Bound and unbound $^3$H dpm are plotted after subtracting dpm obtained in a control reaction lacking mRNA (23 and 15% of maximal bound and unbound dpm, respectively). When biotin-labeled Lys-tRNA$^{Lys}$ is omitted from a translation of mRNA MTKV, binding of $^3$H to the beads is not observed (not shown).

up to 14-fold higher failed to substantially increase the yield of fMVT (data not shown).

*Incorporation and selection of an unnatural amino acid.* Our tetrapeptide synthesis format is directly amenable to many types of initiation and elongation assays, including the testing of unnatural amino acids for incorporation by ribosomes for mechanistic or selection experiments (see Discussion). For example, translation of mRNA mMTKV (Fig. 3) using the substrates [$^3$H]fMet-tRNA$_i^{fMet}$, [$^{14}$C]Thr-tRNA$_3^{Thr}$, biotin-labeled-Lys-tRNA$^{Lys}$ (Promega), and [$^3$H]Val-tRNA$_1^{Val}$ yielded peptide product containing both biotin and [$^3$H]valine, as judged by product copurification with soft avidin beads (Promega) in a manner dependent on a lysine codon in the mRNA and on biotin-labeled Lys-tRNA$^{Lys}$ (Fig. 8). The reduced yield of fMTbKV relative to fMTV is probably due either to a subsaturating concentration of biotin-labeled Lys-tRNA$^{Lys}$ or contamination of the commercial biotin-labeled Lys-tRNA$^{Lys}$ with Lys-tRNA$^{Lys}$ (see Materials and Methods; the resulting positively charged fMTKV product would not be detected in the assay because of its affinity for the cation-exchange column). This experiment demonstrates that the purified translation system is capable of incorporating an easily selectable, large, unnatural amino acid.

## DISCUSSION

In this study, His-tagged versions of five *E. coli* translation factors have been used to reconstitute a purified oligopeptide synthesis system. These factors are expressed at very high levels in bacteria, are readily purified to near homogeneity in a single affinity chromatography step, and have activities in standard factor assays comparable to those reported for the native factors. Although there is a wealth of three-dimensional structure–function information available for tRNAs, elongation factors, termination factors, and ribosomes, a high-resolution structure of a whole initiation factor has been reported only for IF1 (2). Now that large quantities of all initiation factors can be readily purified, structural studies both alone and in complex with ligands or ribosomal subunits should be facilitated, as illustrated by the solving of the *Thermus thermophilus* IF1-30S structure (50). These structures should assist efforts in the elucidation of general principles of translation initiation and in eubacterial-specific drug design.

Reconstitution of oligopeptide synthesis using either four or five His-tagged initiation and elongation factors only requires addition of ribosomes, mRNA, and aminoacyl tRNAs. The main value of this approach lies in the simplicity of the reconstitution: all factors are easily prepared, and it is unnecessary to add additional elongation factors (8, 9), termination factors, or aminoacyl tRNA synthetases. The components now limiting scale-up are no longer the protein components, but rather, the pure aminoacyl tRNAs. Few purified tRNA isoacceptors are available commercially, and purification procedures for the separation of the many closely related isoacceptors are difficult. Large amounts of charged tRNA might be prepared by charging deacylated total tRNAs *in vitro* or by isolation of total aminoacyl tRNA from cells. Alternatively, unmodified *in vitro*-transcribed tRNAs might be aminoacylated and then tested for activity, although some unmodified tRNAs have altered codon-recognition properties (51). Since the testing of unmodified tRNAs in crude translation systems is complicated by the possibility of tRNA modifications *in situ* before translation (52), our purified system (which should lack such modification activities) may prove superior for the testing of unmodified tRNAs and the further elucidation of the roles of modified bases in codon recognition and translational frameshifting (53).

We have provided further evidence that the reconstitution of a purified oligopeptide synthesis system requires particular attention to the purity of the ribosomes (27), since only highly purified ribosomes result

in strong translation factor dependencies. HPLC analysis of tetrapeptide synthesis demonstrated that 80% of the oligopeptide products are full length and the remaining 20% result from pausing or premature termination. This observation shows that the early steps of the translation of our prototype template are predominantly, but not completely, processive. Multiple oligopeptide products shorter than full length have also been detected in a purified hexapeptide translation system (9). The incomplete processivity is reminiscent of the early steps in transcription. However, transcription initiation is far less processive because it primarily yields small premature termination oligonucleotide products (28).

The role of the epsilon translation enhancer sequence, a common element of *E. coli* expression vectors, is controversial. Because the sequence has previously been reported to be either very stimulatory (18), or inactive (19) *in vivo*, we evaluated this sequence in a purified translation system. Scrambling or deleting the native epsilon sequence reduced the efficiency of the early steps of translation, supporting its designation as an enhancer and indicating that it does not function through interaction with a termination factor or an unknown translation factor. Since partial stimulation of translation occurs when a scrambled variant of the U-rich epsilon sequence is included, the precise nine-nucleotide sequence of epsilon is not essential for activity. Although the mechanism of enhancement by this sequence is unknown, proposals include binding to 16S ribosomal RNA by base pairing (18) and binding to ribosomal protein S1 (see discussion in 54). Indeed, the ribosomal footprint on mRNAs extends to nucleotide −39 (55), well beyond the S-D sequence and the 5′ termini of all our mRNAs. More generally, this purified initiation and translation system should simplify the study of the several reported translation enhancer sequences (54) and of other aspects of initiation and early elongation such as the less well-established factors (8, 9, 56, 57) and potential new factors.

Ultimately, this simplified translation system may form the core "protein polymerase" of reconstituted purified systems to study vectorial processes, such as chaperone-catalyzed protein folding (58), protein deformylation and removal of the N-terminal methionine (59), and the mRNA degradosome (60). The system may have certain advantages over existing chemical, *in vivo*, or crude *in vitro* peptide or peptidomimetic synthesis systems (e.g., 61), such as enabling quantitative incorporation of several different genetically encoded unnatural amino acids (without interference from endogenous amino acids and aminoacyl tRNA synthetases) for mechanistic studies or the generation of peptidomimetic libraries. Ribosome display (46) and mRNA-puromycin display (62, 63) could be improved by preventing interference during translation from endogenous termination factors or nucleases (34; mRNA is not degraded in our system). The incorporation and selection of an unnatural amino acid using our purified system is the first step toward the creation of a system for "pure translation display" to generate extremely diverse libraries of genetically encoded peptidomimetics which could then be subjected to directed Darwinian evolution ("peptidomimetic evolution") for ligand and drug discovery.

## ACKNOWLEDGMENTS

We are very grateful to F. Nallaseth for demonstration of the dipeptide reaction, [$^3$H]fMet-tRNA$_i^{fMet}$, and discussions; H. Qu for overexpression and purification of IF1H and EF-TuH; F. Etienne for comparative dipeptide assays and [$^3$H]fMet-tRNA$_i^{fMet}$; and Y.-W. Hwang and D. Miller for the unpublished EF-TuH clone. We also thank V. Cornish, M. Ehrenberg, U. RajBhandary, and B. Reid for helpful discussions; D. Baronas-Lowell for materials and detailed protocols; and N. Brot, G. Chen, M. Grunberg-Manago, C. Gualerzi, R. Himes, J. Horowitz, K. Kounga, S. Laalami, H. Noller, L. Roner, A. Savelsbergh, I. Schwartz, R. Spurio, K. Wilson, and W. Wintermeyer for materials (see Materials and Methods).

*Note added in proof.* While this manuscript was in press, we learned that the synthetase-dependent pure translation system (6, 7) has been reconstituted using epsilon and His-tagged factors and synthetases (Shimizu *et al. Nat. Biotechnol.* **19,** 751–755, 2001). A stop codon was recruited to incorporate valine efficiently using a chemically charged suppressor tRNA.

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